STUDIES TO DETECT ANTIBODIES IN RABBITS FOLLOWING THE INJECTION OF EMBRYO-BREI

by

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INTRODUCTION

Embryonic tissue has been reported to be antigenic as determined by transplantation immunity. These reactions have been graded as first set and second set rejections of homografts. Circulating antibodies have been demonstrated in second set rejection but not in first set rejection. The ability of the embryo to act as a homograft indicates that an antigen-antibody reaction is involved. If several pregnancies occurred consecutively, a constant source of antigenic stimuli represented by the embryos could possibly induce an immunological reaction in the female.

The gestational period of mammals provides sufficient time for the dam to react adversely to the developing tissue of paternal origin. A lack of response indicates that either no immunological stimulation occurred or possibly the response was occluded by the trophoblastic cellular stratum. Brambell et al. (1951) established that the antibodies present in maternal blood plasma were very similar to those found in the developing embryo's yolk-sac fluid. A selective buffer zone interfering with passage of harmful antibodies to the embryos might exhibit a protective mechanism which would allow the graft to be maintained. If this selective bio-mechanism were disrupted, exposure of embryonic tissue to the dam could possibly cause embryonic loss.

Since sensitive serological tests have not been employed for determining the response of the dam to embryos, this study was undertaken to determine if injections of such embryonic tissues into the dam would stimulate the production of anti-embryonic antibodies. Continued antigenic

stimulation should increase the antibody titer to embryonic brei so that it could be determined by microassay procedures. Several immunological tests were conducted for the detection of extremely small amounts of antibody produced by the dam in response to her embryos.

REVIEW OF LITERATURE

Transplantation Immunity

The inability of fetal tissue to induce transplantation immunity has led authors to speculate as to why a rejection of this homograft does not occur. Medawar (1953) proposed three possible explanations for the persistence of the graft: (a) antigenic immaturity of the embryo, (b) immunological inertness of the mother, and (c) anatomical separation of the embryo from the mother. Woodruff (1958) conducted two experiments and rejected the first two explanations offered by Medawar. The graft rejections in experimental animals were the bases for rejection of these hypotheses. Transplantation immunity was induced in the mothers by skin grafts from the father's tissue prior to mating. This induced a first set rejection and would determine that the mother was not immunologically inert. Fifteen days after mating, two fetal hindquarters from each pregnant doe were, respectively, intramuscularly grafted in each doe. The control group which did not receive the skin grafts but only nonhomologous fetal hindquarters showed first set homograft rejection, whereas the animals in the immunized group demonstrated second set rejections. The results indicated that fetal tissues were antigenically competent as noted by the second set graft rejection. The animals in both experimental groups were immunologically competent as

indicated by either first or second set rejections. The latter of the three explanations was chosen as the decisive factor by Woodruff (1958).

The ability of antibodies to interfere with or in any manner disrupt normal pregnancy has been investigated by Cohen and Nedzel, 1940; Seegal and Loeb, 1945; Katsh and Bishop, 1958; Isogima et al., 1959; Katsh, 1959; Kiddy et al., 1959; Tyler and Bishop, 1961; Brent et al., 1961; Lanman et al., 1962; Menge et al., 1962; Simmons and Russel, 1963; Hašková, 1963; Otani et al., 1964; Singh, 1965; and Brent and Johnson, 1967. Results obtained by these workers indicated that a malformation occurred inhibiting placental function. Direct evidence supporting interference of pregnancy was presented by Cohen and Nedzel (1940) who induced abortion in pregnant guinea pigs using anti-placental sera. Seegal and Loeb (1945) reported anti-rat placental serum rabbit origin caused rats to resorb developing fetuses. Uhr and Anderson (1962) found placental tissue to be antigenic for parental mice. Hašková (1963) modified her earlier findings by stating transplantation immunity was dependent on strain-combination relationship between parental strains of mice.

The possibility exists that semen could induce transplantation immunity after formation of the zygote. Semen was determined to be antigenic by Katsh and Bishop, 1958; Isojima et al., 1959; Katsh, 1959; Kiddy et al., 1959; and Tyler and Bishop, 1961. Menge et al. (1962) concluded that anti-rabbit semen sera of cattle origin inhibited development of one-day-old embryos. Katsh (1959a) reported that immunization with homologous sperm was injurious for pregnant guinea pigs by inhibiting placental function.

Transplantation antigens can excite the formation of humoral antibodies, and the ability of these antigens to inhibit agglutination or

lysis by specific absorptions was noted by Brent et al. (1961). Singh (1965) found that intravenous injections with bovine embryonic brei in the bovine resulted in a very low titer of circulating antibodies. Billingham et al. (1956) suggested that DNA proteins provided adequate antigenic stimuli to induce skin transplantation immunity. Snell (1957) stated that these results could have been due to an adjuvant effect.

The ability of the trophoblastic cells to possibly act as a bio-selective membrane causing the dam to be immunological inactive was noted by Lanman et al. (1962). They reported that trophoblastic giant cells lack antigens. Simmons and Russell (1963) determined that a lack of antigenic stimulus from trophoblastic giant cells was responsible for the persistence of the embryos. Hybrid ova resulted in prolonged survival with isologous and homologous grafts in experimental animals. Transplantation immunity directed against the ova transplants was relatively ineffective. Schlesinger (1964) used a hemagglutinating system to determine that certain placental tissues were antigenic, but the trophoblastic cells did not possess histocompatibility antigens.

Brent and Johnson (1967) indicated that heterologous anti-rat kidney or placental antisera injected into 8-day pregnant rats produced a high degree of deformity. The yolk sac basement membrane, used as the antigen, was purified by sonication and trypsinization. The teratogenic inducing sera interfered with yolk sac function of the embryos for the above injected rats.

Hašková (1961) reported that transplantation immunity was not invoked when placentas were subcutaneously injected into the maternal strain. Ketchel et al. (1966) noted that pregnancies were not disturbed

when females were injected with fetal placental tissue. Homogenized tissues (fetal placentas, fetal tissue, and adult spleen), whole cells of fetal placentas, and cells of fetal placentas incorporated in Freund's complete adjuvant were injected into respective groups of mice. The ability of the mice to maintain normal pregnancy indicated no disturbance of placental function.

Adjuvants

Methods to enhance antibody formation have been used for a considerable period of time. Adjuvants have been needed to increase the immunizing capacity for serum proteins, pollens, bacterial cells, viruses, and toxoids. Landsteiner and Simms, 1923; Schultz and Swift, 1934; Swift and Schultz, 1936; Freund and Bonanto, 1944; Landsteiner and Chase, 1941; Friedewald, 1944; and Freund et al., 1948 reported successful usage of adjuvants for a wide spectrum of proteinous material. A lipid extract of Mycobacterium tuberculosis, paraffin oil, and Arlacel A (mannide monooleate) gave a greater antigenic response as reported by Freund et al. (1951). Emulsification of these constituents with an equal volume of antigenic material increased the antibody titer. This water-in-oil emulsion (Freund's complete adjuvant) gave a prolonged stimulation by forming a depot which slowly released the antigenic material and also protected the antigen against destruction by the host. Emulsions prepared without the lipid extract (Freund's incomplete adjuvant) would still augment antibody formation, but the development of necrotic sites was greatly reduced. Freund (1958) suggested that antibody producing cells accumulate near the administered dose when an adjuvant is used.

Hemagglutination Studies

Boyden (1951) employed sheep erythrocytes treated with a 1-20,000 dilution of tannic acid to adsorb proteineous antigens so they could be titrated by serological tests. The tests were conducted according to the following procedure. Erythrocytes were prepared by adding a 2.5% suspension to the diluted tannic acid by adding equal volumes and incubating for 10 min at 37 C. The washed and resuspended tanned erthrocytes were added to dissolved tuberculin purified protein derivative (PPD) in buffered saline (pH 6.4) in ratio 1-4 and incubated for 15 min at room temperature. The mixture was centrifuged 2 times and finally resuspended in 1 volume of 1-250 normal rabbit serum (NRS) diluent. This diluent was used because autoagglutination was greatly decreased. Sera for titration were inactivated by heating at 56 C for 30 min and were absorbed with equal volume of packed, washed sheep erythrocytes. A serial fourfold dilution of the antiserum in 1-100 NRS in buffered saline (pH 7.2) was titrated by adding a drop (0.05 ml) of sensitized tannic acid treated erythrocytes to each test tube. The tubes were mildly agitated for 30 min and after 5 hr at room temperature placed in a refrigerator at 0-4 C. After overnight incubation and 5 min at room temperature, readings were made by gently shaking the tubes to observe clumping. He noted that other antigens such as ovalbumin and serum proteins could be titrated by this tannic acid procedure. Complement titration was attempted but hemolysis occurred in all tubes because the tannic acid acted as an amboceptor. Stavitsky (1954) found that the passive hemagglutination (PHA) test was 1000 times more sensitive than a ring test.

Flick (1948) used formalized cells (FC) to titrate a virus. This system eliminated numerous variables for his assay procedure. Carter and Rappay (1962, 1963) employed a method described by Csizmas (1960) to prepare FC. These workers were using an hemagglutination (HA) test for typing Pasteurella multocida. Formalized cells were prepared by placing a dialysis sac containing 40% formaldehyde solution in a beaker. The cell suspension was poured over the dialysis sac. This beaker was mechanically agitated for 2 hr after which the contents of the dialysis sac were poured into the beaker. Agitation was continued for 12 to 18 hr. This mixture was filtered through several layers of cotton gauze. Then one-half volume of saline was added to the filtrate, and subsequently, cells were washed 6 times in 10 volumes of saline. The packed cells were finally resuspended in saline to prepare a 50% suspension. These FC were coated with extracted lipopolysaccharide from different serological types of P. multocida. Antigen complexes were titrated with specific antibodies using the indirect hemagglutination test (HAI). Ten μ g/ml of lipopolysaccharide was the amount required for maximal HA patterns.

George and Vaughan (1960) indicated the importance of antigen preparation in conducting PHA tests. Denaturation of egg albumin increased its affinity for tanned cells giving the cells stronger attraction for the proteinous material. These authors emphasized that proteins other than egg albumin may react in a similar fashion.

Consideration has been given to the method of formalizing cells in conjunction with tanning them. Cole and Farrell, 1955; Fulthorpe, 1957; Weinbach, 1958; Fulthorpe et al., 1961; Daniel et al., 1963; and Whitman and Hetrick, 1965 found that formalized tannic acid treated cells (FTC)

increased the longevity of these cells and reduced nonspecific hemolysis.

Daniel et al. (1963) investigated the inconsistent results obtained when using formalized, protein sensitized erythrocytes. These workers noted that the variable results obtained in using freshly sensitized erythrocytes prompted the use of formalized erythrocytes. Variables determined for FTC were concentration of tannic acid, time of exposure for tannic acid, and pH of the titration. Greater stability and longevity were noted when using FTC instead of freshly prepared tanned cells. The mechanism for antigen adsorption to tanned cells was not determined.

Whitman and Hetrick (1965) followed Weinbach's (1958) procedure for preparing FTC to be used for HAI. Sheep erythrocytes were formalized by mixing equal volumes of a 3% formaldehyde solution with 8% erythrocyte suspension. This suspension was incubated for 20 hr at 37 C, after which formalized cells were repeatedly washed to remove excess formalin. Tannic acid treatment was accomplished as follows. A 10% suspension of formalized cells was added to an equal volume of a 1-20,000 dilution of tannic acid (pH 7.2). This suspension was incubated in a 37 C water bath for 15 min with periodic agitation. The cells were washed with saline (pH 7.2) and stored at 4 C until used.

One ml of FTC was mixed with 2 ml of undiluted stock of infectious bovine rhinotracheitis virus and 2 ml of pH 6.4 buffered saline. After a 15 min incubation period in a 37 C water bath, the suspension was centrifuged and washed with serum diluent 2 times. This diluent was prepared by adding 1 ml of inactivated NRS absorbed with FTC to 99 ml of pH 7.2 buffered saline.

Passive Cutaneous Anaphylaxis

Ramsdall (1928) reported that passive sensitization in the guinea pig was easily induced by using horse serum. Primary injection of homologous anti-horse serum of rabbit origin was subcutaneously administered at the base of the guinea pig's ear. A secondary injection, consisting of horse serum and 0.2% solution of trypan blue was given intravenously in the marginal ear vein after sufficient time to allow for the antibody to be fixed to cellular tissues. A uniform change from redness to intense blueing marked the injected site.

Ovary (1958) reported that 3 to 6 hr after intradermal injection of antiserum, administration of antigen-dye mixture intravenously caused a marked blueing of the injected area. Ovary and Bier (1952) studied the relationship between the Arthus phenomenon and passive cutaneous anaphylaxis. These workers indicated that the differences between the two reactions were dependent upon their mode of action. The Arthus reaction was due exclusively to a damaging effect of the circulating antibody reacting with locally fixed antigen by forming complexes injurious to small blood vessels. The passive cutaneous anaphylactic reaction was reversed in that the antibody would be fixed and circulating antigen would be present. The cutaneous reaction would cause histamine release for local anaphylaxis whereas the Arthus would be local necrotic-hemorrhagic type.

Ovary (1965) considered the postulation that a structural affinity for antibodies existed for skin sensitization and transplacental passage. He noted that the fixation of the antibody to rabbits' tissues was poor,

and the placental tissues would be peculiar if they possessed a fixation site different from other tissues.

MATERIALS AND METHODS

Removal of Embryos

Doe rabbits from 6 to 9 months old were randomly selected and serviced twice with different bucks. The does were palpated during pregnancy to check for developing embryos.

Prior to surgery rabbits were removed from feed and water. The females were anesthetized by injecting into the marginal ear vein 2.5 to 5 grains pentobarbital sodium (dosage depending on weight of rabbit) diluted equal volumes with physiological saline. Rabbits were clipped and surgically scrubbed. Caesarean section by means of a ventral midline incision in dorsal recumbency was the operational approach to remove embryos, and the uteri were exteriorized through the incision. A stab incision was made through the uterine wall above each embryo. Embryos were removed through this incision by slight tactile pressure and collected in sterile sample containers. These samples were immediately placed in a freezer at -18 C. After removal of embryos, the uterus readily contracted and was manually replaced into normal position. The peritoneum and rectus abdominis muscle were closed with a simple continuous pattern with chromic 2-0 catgut. The skin was sutured with #32 stainless steel wire sutures using a simple interrupted everting mattress pattern. The rabbits were injected intra-muscularly with 100,000 units of penicillin after the surgery.

Preparation of Embryonic Brei

The frozen samples were stored in 2 groups. Embryos from rabbits of Group I were kept several months, whereas, those from Group II were stored from 5 to 30 days. Embryos were thawed at room temperature and added to a pint jar containing 150 ml of physiological saline. By means of an Osterizer head, a Waring blender was used to homogenize the tissues from 1 to 2 min to produce the embryonic brei. Care was taken not to increase the temperature or create excess foam in preparing the brei. This suspension was cultured for sterility on blood agar plates, Sabouraud's agar, and in thioglycollate broth. The preparations were preserved by adding formalin to a final concentration of 0.5%. Ten ml samples were transferred to each of 2 sterile, rubber stoppered, 30 ml vaccine bottles and the remainder of the brei was stored in 160 ml prescription bottles. All samples were placed in the refrigerator at 7 C. Using the micro-Kjeldahl procedure the average nitrogen content of the brei for Group I was 3.6 mg/ml. Equal volumes of supernatant brei and Freund's complete adjuvant (Difco) were emulsified by repeatedly withdrawing and expressing the fluid through an 18 gauge needle fitted to a 10 ml syringe. This water-in-oil emulsion was used in the first series of injections.

In an attempt to increase the titer following the second series of injections, the samples in prescription bottles were alternately frozen and thawed 3 times. Samples were frozen in a Revco deepfreeze (Revco Inc., Deerfield, Mich.) and thawed by placing the sample container under free running cold tap water and then placing the container

in a 37 C water bath. The supernatant brei was incorporated in incomplete adjuvant. This was prepared by adding equal volumes of brei and 1-9 ratio of Arlacel A (Atlas Powder Co., Wilmington, Delaware) and paraffin oil, respectively. A VirTis (The VirTis Co. INC., Yonkers, N. Y.) was used to emulsify the inoculum. These emulsions were checked by microscopic examination of a hanging drop preparation to affirm that the droplets were laked. The emulsions were also dropped on cold water to insure that they were properly incorporated and would not disperse. These emulsions were refrigerated at 7 C and used within 3 to 4 weeks. It was noted that several emulsions did separate after 6 weeks.

Injections of Emulsified Embryonic Brei

Embryonic emulsions were injected into homologous and heterologous does in Group I to determine if sera would have similar titers. In the first series, 3 injections were given per week for a total of 10 injections containing 20 ml of emulsion. These injections were given subcutaneously in the nuchal region. Necrotic tissue developed in this region, therefore subsequent injections followed alternate sites on either side of the dorsal midline. Approximately 6 months later a 4 ml booster shot was given intramuscularly by incorporating equal volumes of brei in incomplete adjuvant.

Rabbits in Group II were injected with a similar schedule as Group I in the first series of injections. Beginning two weeks later they received 3 weekly boosters. These boosters were incorporated in incomplete adjuvant and 8 ml of emulsion were injected for the booster

injections. Quantitative data for antigens are given in Tables 1 and 2. The injection schedules for both groups are given in Tables 3 and 4.

Collection of Antisera

All rabbits of Group I and II were bled approximately 2 weeks after surgery for control sera except does injected with nonhomologous brei in Group I. Sexually immature does were used as sources for normal rabbit sera to be used in preparing the diluent. Sera were collected from Group I rabbits on three separate occasions and from those in Group II on four different bleedings. Animals were removed from feed 12 hr prior to bleeding by cardiac puncture.

The blood was allowed to clot at room temperature and then rimmed. The tubes were centrifuged in an International Refrigerated Centrifuge (International Equipment Co., Boston, Mass.) at 4 C. The serum was transferred to small vials and stored at -18 C until needed. Prior to use the serum was heated at 56 C for 30 min and adsorbed at room temperature with sheep erythrocytes in order to inactivate complement and remove non-specific reactions.

Preparation of Reagents

Buffered Saline. Saline buffered at pH 7.2 was prepared by adding 120 ml of 0.15M KH_2PO_4 and 380 ml of 0.15M Na_2HPO_4 added to 500 ml of 0.85% NaCl.

Formaldehyde. Reagent grade formaldehyde (approximately 40%) was used. The erythrocytes were preserved by adding equal volumes of

Table 1. Quantitative data for antigens from rabbits in Group I.

No. of doe	Date of operation	Age of embryos	No. of embryos removed	No. of unrelated doe injected with embryonic brei	pH of embryonic brei suspension	Nitrogen mg/ml
49	6-65	13	3	50	6.7	4.1
38	8-65	19	12	55	6.8	3.0
44	7-65	21	6	54	6.8	2.7
45	7-65	16	5	47	6.0	3.5
56	8-65	20	10	52	6.5	4.9
37*	8-65	15	8	58	6.5	3.3

* Died after the operation.

Table 2. Quantitative data for antigens from rabbits in Group II.

No. of doe	Date of operation	Age of embryos (days)	Weight of embryos (gms)	pH of embryonic brei suspension
4	1-67	17	11.3	6.7
16	1-67	17	17.1	6.5
39	1-67	17	10.4	6.6
43	1-67	17	15.7	6.7
77*	1-67	17	2.9	6.0
28	12-66	17	30.7	6.7
36	12-66	17	23.2	6.8
60**	12-66	17	20.2	6.6

* Only one embryo removed and it was contaminated.

** Died after the operation.

Table 3. Immunization schedule for rabbits in Group I.

	No. of doe	First injection: Time in weeks I			II			III			IV	Booster+	Bled 1 week after last injection	Bled 10 days after last injection
		ml of emulsion injected												
Homologous brei	49	1	2	2	2	2	2	2	2	3	2*	5		
	38	"	"	"	"	"	"	"	"	"	"	-		
	44	"	"	"	"	"	"	"	"	"	"	-		
	45	"	"	"	"	"	"	"	"	"	"	-		
	56	"	"	"	"	"	"	"	"	"	"	-		
Heterologous brei	50	"	"	"	"	"	"	"	"	"	"	++		
	55	"	"	"	"	"	"	"	"	"	"	-		
	54	"	"	"	"	"	"	"	"	"	"	-		
	47	"	"	"	"	"	"	"	"	"	"	++		
	52	"	"	"	"	"	"	"	"	"	"	-		
	58	"	"	"	"	"	"	"	"	"	"	-		

* Reduced dosage due to necrosis.

\+ Injection given approximately 7 months later.

++ Died prior to immunization and cause of death was not determined.

Table 4. Immunization schedule for rabbits in Group II.

	First Injections[+]									Boosters[++]		
	Time in Weeks											
No. of doe	I			II			III			VI	VII	VIII
	ml of emulsion injected											
4	1	2	2	2	2	2	2	3	2*	2	3	4
16	"	"	"	"	"	"	"	"	"	"	"	"
39	"	"	"	"	"	"	"	"	"	"	"	"
43	"	"	"	"	"	"	"	"	"	"	"	"
77	"	"	"	"	"	"	"	"	"	"	"	"
28	"	"	"	"	"	"	"	"	"	"	"	"
36	"	"	"	"	"	"	"	"	"	"	"	"

[+] Brei emulsified with Freund's complete adjuvant.

[++] Brei emulsified with incomplete adjuvant.

* Dosage was reduced because of necrosis.

a 10% suspension of washed erythrocytes to a 3% formaldehyde solution buffered in pH 7.2 saline.

Method of Formalizing Erythrocytes

Sheep blood was collected on the day of formalization in an equal volume of Alsever's solution and washed and resuspended 2 times in cold physiological saline by centrifuging at 1000 rpm for 10 min in a refrigerated centrifuge (4 C). Inoculation of cells into nutrient broth showed the cells to be sterile. Weinbach's (1958) method of formalizing the erythrocytes was accomplished as follows. An 8% suspension of sheep erythrocytes plus 3% HCHO solution were mixed with equal volumes and incubated for 25 hr at 37 C. The formalized cells were washed 3 times in saline to remove excess HCHO. An equal volume of formalized cells were added to 1-20,000 dilution of freshly prepared tannic acid (pH 7.2). This suspension was incubated in a 37 C water bath for 15 min with occasional shaking. The erythrocytes were then washed 2 times with a 1-100 dilution of normal rabbit serum (NRS) in buffered saline (pH 7.2) and resuspended to make a 10% suspension.

Five hundred ml of FTC were prepared and frozen at -18 C until needed. Cells were removed from the freezer and placed under cold running tap water to facilitate thawing. These FTC were then placed in a 37 C water bath to complete thawing. The FTC had a tendency to form auto-aggregates in very small complexes after the third thawing. A 10 ml syringe with an 18 gauge needle was used to withdraw and express the FTC several times to separate the aggregates. Thawed

cells were kept for a 2 week period in the refrigerator at 7 C and the unused portion was discarded.

Sensitization of Formalized Erythrocytes

The supernatant fluid of the embryonic brei suspension was used to coat the FTC on the day of titration. A modification of Boyden's (1951) procedure was used in conducting the microassay titrations. The method is given in detail. One ml of embryonic brei fluid, 2 ml of 1-100 NRS pH 7.2 buffered saline (diluent), and 1 ml of 10% suspension of FTC were mixed and incubated for 30 to 45 min in a 37 C water bath with periodic agitation. This suspension was centrifuged at 1000 rpm for 8 to 10 min in an angle head centrifuge at 4 C. The supernatant fluid was decanted, and if this fluid was turbid the cells were again washed in diluent equal to the original volume. After the final washing the supernatant fluid was decanted. Ten ml of diluent were added to the centrifuged FTC-antigen complex. A lab-mixer (Aloe Scientific, St. Louis 3, Mo.) was used to resuspend the complexes. These cells were ready to be used in PHA tests.

Passive Hemagglutination Test

Serial twofold dilutions of antiserum were made in wells of disposo-trays (The Chemical Rubber Co., Cleveland 14, Ohio). Antiserum for the diluent was absorbed with washed, packed sheep RBCs to remove non-specific reactants. To each well 0.5 ml of the prepared

FTC-antigen coated cells was added, and wells were mixed by gentle agitation. The protocol for the test is given in the Appendix.

A preliminary titration using various dilutions of the brei was made to determine the amount needed to coat the cells. It was found that the undiluted brei suspension gave the best HA pattern. Room and refrigerated temperatures were used to determine the best incubation temperature for the development of HA patterns. It was found that overnight incubation in the refrigerator at 7 C would give the best HA pattern. The tests were removed from the refrigerator, and the patterns became more marked. Any disruption caused the complexes to dissociate and form a compact button. Prolonged incubation at room temperature after removing from the refrigerator caused all wells to show a button pattern.

The cellular patterns were graded after overnight refrigeration and approximately 1 hr at room temperature as follows:

4+ = lattice cellular aggregate

3+ = smooth mat on bottom of tubes with folded edges

2+ = smooth mat, intermittent breaks in folded edge

1+ = well marked with circular pattern but having a slight mat

$\pm$ = dark circular ring on the periphery of a small mat

\- = discrete circular button in center of the well

Other Immunological Studies

Passive Hemagglutination Test. Boyden's (1951) passive HA test was conducted to determine antibody titer. The procedure was as follows: 3 ml of defibrinated sheep blood were added to 10 ml of saline in a 15 ml tube. These cells were washed 3 times at 1000 rpm for 10 min in a Servall centrifuge (Ivan Servall Inc., Norwalk, Conn.). Then 0.5 ml of packed cells was added to saline to make 2.5% suspension. This suspension was diluted equal volume with 1-20,000 dilution of tannic acid. The tannic acid was freshly prepared and stored in the refrigerator at 7 C until needed. This mixture was incubated for 10 min in a 37 C water bath. Cells were repeatedly washed and resuspended 2 times in diluent equal to the original volume. After the third centrifugation the supernatant was discarded, and these tanned erythrocytes were coated with embryonic brei by pipetting 4 ml of pH 6.4 buffered saline into 1 ml of packed tanned cells. One ml of embryonic brei fluid was added to form TC-ag (tannic acid treated antigen coated cells). TC-ag mixture was incubated for 30 to 45 min in a 37 C water bath. Control erythrocytes were prepared by adding saline instead of antigen. Cells were centrifuged and the supernatant decanted. The filtrates were resuspended in 6 ml of the diluent, then centrifuged and the supernatant discarded. The filtrates were finally resuspended in 10 ml of the diluent and were used within an 18 hr period.

Antiserum from each doe was serially titrated by two-fold dilutions in diluent using test tubes (13 x 100 mm). To the appropriate tubes,

0.05 ml of TC-ag was added. The tubes were agitated to insure suspension and mixing of reagents. Tests were incubated overnight at room temperature and then read. The protocol used for this test is given in the Appendix.

Gel Diffusion. Micro tests using microscope slides were performed by Ouchterlony's (1949) method of two dimensional gel diffusion. Embryonic brei fluid was added to the outer wells in the following dilutions: undiluted, 1-10, 1-25, and 1-50 with undiluted antibody in the center well.

Ring Precipitin Test. Dreyer tubes were half filled with antiserum using a Pasteur pipette. Various dilutions (undiluted to 1-512) of antigen were overlaid on the antiserum. Tests were checked for a precipitin ring at 15 min intervals for 1.5 hr. Tests were refrigerated at 4 C overnight and again checked for a precipitin ring.

Passive Cutaneous Anaphylaxis. Guinea pigs were prepared for passive cutaneous anaphylaxis (PCA) by using a depilatory agent 24 hr prior to testing. After this period two sites were injected with undiluted and diluted antiembryonic sera produced in rabbits. A third injection used saline for a control. In performing the intracutaneous injections into the skin, care was taken not to pinch the skin. A sharp 27 gauge needle was administered parallel to the forefinger and 0.1 ml of antiserum was injected. The same volume was given in each of the three injected sites located in the mid-dorsal region of the guinea pig. After allowing 3 hr for fixation of the antibody, 0.5 ml

embryonic brei plus 0.5 ml of 0.1% Evans blue were injected into either the marginal ear vein or the heart.

The injected sites were watched for 3 hr to determine if any change occurred. Several animals were sacrificed at the end of the 3 hr period, and the skin of the back was exposed to facilitate reading the reaction.

RESULTS AND DISCUSSION

The presence of anti-embryonic antibodies was found in the sera from 12 rabbits injected with homologous brei incorporated in adjuvants. The sera from 6 rabbits injected with heterologous brei incorporated in adjuvants gave a similar titer. Boyden's (1951) passive hemagglutination test modified by Weinbach (1958) detected the antibodies. Following triplicate titrations employing the FTC-antigen complexes, the test showed positive HA patterns in dilutions as high as 1-512. The wells illustrated in Plate I, Fig. 1, 2, and 3 show the positive hemagglutinating patterns. Non-specific HA patterns were also noted in the sera of rabbits bled prior to immunization. This could possibly be due to the delay of approximately 2 weeks after surgery and prior to bleeding giving the doe sufficient time to be exposed to any remaining embryonic fluid or tissues. The hemagglutinating patterns are recorded in the Appendix.

The sera obtained after the different gestational periods were found to have similar titers. Older embryos were well developed by 20 days and possessed tissues that were probably antigenic. To overcome this factor embryos were removed after 17 days. The prolonged

EXPLANATION OF PLATE I

Fig. 1. Row A is control serum (collected prior to immunization) for rabbit 4 showing negative HA pattern. Well A-C is control for serum used for diluent. Row B gives titer after first bleeding and C shows increase in titer for second bleeding. Rows D, E, and F show similar results for rabbit 43. Well G-U is the control for FTC. Tests are recorded in the Appendix.

Fig. 2. Titrated same as above. Rows A, B, and C are results from rabbit 16. Rowd D, E, and F give titers for rabbit 40. Antigen-antibody complexes are dissociating due to prolong incubation time. Patterns were recorded prior to elution.

Fig. 3. Titrated same as above. Rows A, B, and C are results from rabbit 28. Rows D, E, and F show the pattern for rabbit 39.

PLATE I

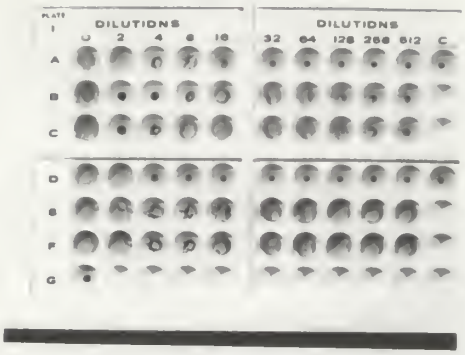

Fig. 1

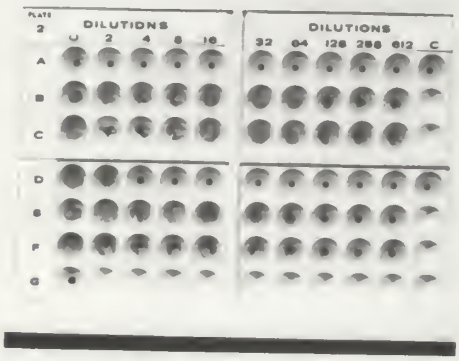

Fig. 2

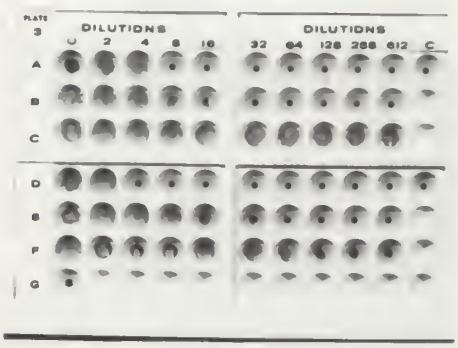

Fig. 3

adsorption time for embryos under natural condition would vary greatly, but they could still be antigenic. The titer for the control rabbits in Group I injected with heterologous brei was similar to those receiving homologous injections indicating the similarity for the source of the antigenic material (Plate II, Fig. 3).

Ovary (1965) noted that 19S antibodies did not induce a PCA reaction. He disagreed with Brambell (1963) that the fixation sites for sensitization in the skin would be structurally identical with those sites present in the placenta. Transplacental passage could still occur although the PCA reaction was not induced.

Boyden's (1951) PHA test with tanned cells gave sporadic results or were not readable. Formalized tannic acid treated cells had a greater longevity, were more stable, and gave a reproducible titer. These were the reasons for preparing FTC and adsorbing the cells with brei for titration. The prepared cells were alternately frozen at -18 C and thawed at room temperature four times. That this procedure did not cause any change in the cells can be observed in the photographs of stained (Wright's stain) and unstained cells shown in Plate II, Fig. 1 and 2. After the third thawing cells would still adsorb brei but on the fourth thawing sensitivity was lost and brei was not adsorbed. It may be possible to coat the FTC with proteinous materials and freeze the FTC-ag complexes. This would save time instead of preparing the cells each time.

Precipitin tests using gel diffusion and the interfacial ring test were negative for the detection of anti-embryonic antibodies.

EXPLANATION OF PLATE II

Fig. 1. FTC stained with Wright's stain.

Fig. 2. FTC unstained (1000X).

Fig. 3. Rows A (not immunized, sexually mature doe), B (control NR5), and E (absorbed antiserum for doe 54) are controls. Row D indicates anti-embryonic antibodies against heterologous brei for rabbit 54. Row C indicates a slight cross reaction with serum from rabbit 47.

PLATE II

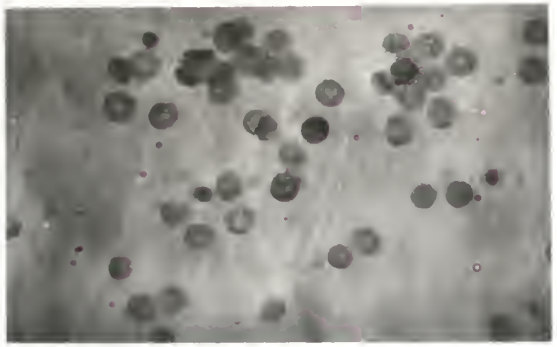

Fig. 1

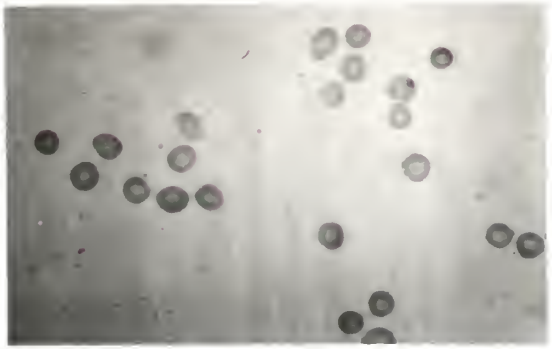

Fig. 2

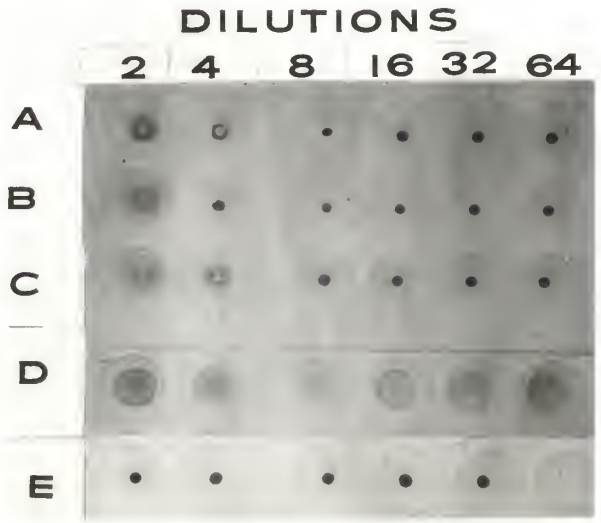

Fig. 3

Stavitsky (1954) reported that tanned cells were 1000 times more sensitive than the ring test and equal to a skin sensitization test. PCA tests, as described by Ovary (1958), were attempted and interpreted to be negative using anti-embryonic serum from rabbits of Group II. Two guinea pigs, injected with sera from rabbits giving the highest HA patterns, demonstrated a definite redness at the injected sites but since there was no blueing the test was considered negative.

The passive hemagglutination test indicated the possibility for antibody nitrogen to be slightly greater than 0.003 μ gN/ml. The possibility of these antibodies disrupting normal pregnancy could not be determined at this time. Their existence would imply a reaction is possible and in all probability may be harmful to the developing embryos. If reduction of fertility due to antigen-antibody reactions does occur, this test might be useful in detecting a reduced fertility potential of a breeder. More studies would be necessary to determine if this relationship would hold true.

SUMMARY

Specific antibodies were detected in the sera from 18 rabbits injected with homologous or heterologous brei incorporated in adjuvants. Formalized tannic acid treated cells adsorbed with embryonic brei titrated with the homologous antiserum was the serological procedure employed. This titration system was very stable and gave reproducible results.

PHA tests using tanned cells gave a positive test, but non-specific hemolysis caused sporadic results. PCA tests induced a redness in 2 of the 7 guinea pigs injected with serum from rabbits of Group II. The tests were considered to be negative because no blueing occurred at the injected sites. Gel diffusion and interfacial ring tests did not detect any serological response. The ability of the antibodies produced to interfere with normal pregnancy was not determined.

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APPENDIX

Protocol For Passive HA Test Using Formalized Erythrocytes

Well No.	1	2	3	4	5	6	7	8	9	10	11
NRS diluent	---	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5*	0.5
Diluted ab before immunization	0.5	0.5	(serially diluted 0.5)								---
FTC-AG coated	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
NRS diluent	---	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5*	
Diluted ab after 1st and 2nd immunization	0.5	0.5	(serially diluted 0.5)								
FTC-Ag coated	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	

* Discard 0.5 ml.

(0.5 ml control RBC and 0.5 ml antibody in well G-U).

Protocol For Boyden's Passive HA Test

Tube No.	1	2	3	4	5	6	7	8	9	10
NRS diluent	---	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	----
Diluted ab	0.5	0.5	(serially diluted 0.5)						----	0.5
TC-ag	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	----
Control RBC	----	----	----	----	----	----	----	----	----	0.05

Titer For Anti-embryonic Antibodies For Group II

Rabbit No.	Reciprocal of Titer									
	Undiluted	2	4	8	16	32	64	128	256	512
16	4+	4+	4+	4+	4+	4+	4+	2+	1+	±
43	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
36	4+	4+	4+	4+	3+	2+	1+	1+	1+	-
39	4+	4+	4+	4+	4+	4+	3+	-	-	-
40*	4+	4+	4+	4+	4+	4+	±	-	-	-
4	4+	4+	4+	4+	4+	4+	2+	1+	±	-
28	4+	4+	4+	4+	4+	4+	4+	4+	3+	3+

* Embryos injected were from a heterologous doe. Rabbit No. 77 was not recorded because the embryo was contaminated.

Titer For Anti-embryonic Antibodies For Group I

Brei Injected Into Homologous Rabbits

Rabbit No.	Undiluted	2	4	8	16	32	64	128	256	512
49	4+	4+	4+	4+	3+	2+	1+	-	-	-
38	4+	4+	3+	2+	1+	1+	1+	-	-	-
44	4+	4+	4+	4+	4+	1+	-	-	-	-
45	4+	4+	4+	4+	3+	3+	2+	1+	-	-
56	4+	4+	4+	3+	3+	2+	1+	-	-	-

Brei Injected Into Heterologous Rabbits

Rabbit No.	Undiluted	2	4	8	16	32	64	128	256	512
50	4+	4+	4+	4+	4+	4+	3+	2+	-	-
55	4+	4+	4+	4+	3+	1+	1+	-	-	-
54	4+	4+	4+	4+	3+	2+	1+	-	-	-
47	4+	4+	4+	4+	4+	4+	3+	±	-	-
52	4+	4+	4+	4+	3+	2+	1+	-	-	-
58	4+	4+	4+	4+	3+	3+	1+	±		

STUDIES TO DETECT ANTIBODIES IN RABBITS FOLLOWING THE INJECTION OF EMBRYO-BREI

by

DONALD R. CHILGREEN

B. A., Marion College, 1964
Marion, Indiana

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

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Manhattan, Kansas

1967

Embryonic brei was produced by removing embryos by Caesarean section and homogenizing the tissues in physiological saline by using a Waring blender. The brei was cultured in various media to affirm that it was sterile. Supernatant brei was emulsified with an equal volume of adjuvants to be injected into heterologous and homologous does to determine if anti-embryonic antibodies could be detected by serological procedures.

The various immunological systems employed were the passive hemagglutination test, passive cutaneous anaphylaxis, the ring precipitin test, and the gel diffusion test. Positive results were obtained by employing formalized tannic acid treated cells in the passive hemagglutination test. Eighteen rabbits were used and all animals demonstrated the presence of anti-embryonic antibodies in their serum when this method was used. The other tests employed gave negative results.

General properties of the antibodies were not determined. The passive hemagglutination test was very sensitive and detected a minute amount of antibody. It was not determined if the presence of these antibodies could interfere with normal embryo development.